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(57) Abstract

We have now discovered a novel class of antiangiogenic proteins and peptides. Some of the proteins are more potent antiangiogenic agents than presently known proteins such as angiostatin and endostatin. The proteins that can be antiangiogenic agents include those of SEQ ID NOS:1, 2 and 3. We have also discovered pharmaceutical compositions containing an angiogenic inhibitory polypeptide or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.

(57) Abrégé

La présente invention concerne la découverte d'une nouvelle classe de protéines et de peptides anti-angiogéniques. Certaines de ces protéines constituent des agents anti-angiogéniques plus puissants que les protéines connues à ce jour telles que l'angiostatine ou l'endostatine. Les protéines pouvant agir comme anti-angiogéniques sont celles de SEQ ID NOS:1, 2 et 3. L'invention porte également sur des compositions pharmaceutiques renfermant un polypeptide inhibiteur de l'angiogenèse ou des acides nucléiques codant pour ce polypeptide qui, en doses thérapeutiquement efficaces, sont capables d'inhiber la prolifération de cellules endothéliales. Sont également décrites les méthodes d'utilisation de ces compositions.

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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS

(57) Abstract

We have now discovered a novel class of antiangiogenic proteins and peptides. Some of the proteins are more potent antiangiogenic agents than presently known proteins such as angiostatin and endostatin. The proteins that can be antiangiogenic agents include those of SEQ ID NOS:1, 2 and 3. We have also discovered pharmaceutical compositions containing an angiogenic inhibitory polypeptide or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.

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Description

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METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention provides for a novel pharmaceutical composition, and method of use thereof for treatment of diseases or disorders involving abnormal angiogenesis.

2. Background

Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products are removed from living tissue. Angiogenesis refers to the process by which new blood vessels are formed. See, for example, the review by Folkman and Shing, *J. Biol. Chem.* 267 (16), 10931-10934 (1992). Thus, where appropriate, angiogenesis is a critical biological process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe negative consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors have a sufficient supply of oxygen and nutrients that permit it to grow rapidly and metastasize. Because maintaining the rate of angiogenesis in its proper equilibrium is so critical to a range of functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteases secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space, then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

In adults, the proliferation rate of endothelial cells is typically low compared to other cell types in the body. The turnover time of these cells

can exceed one thousand days. Physiological exceptions in which angiogenesis results in rapid proliferation typically occurs under tight regulation, such as found in the female reproduction system and during wound healing.

The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (Folkman, *N. Engl. J. Med.*, 285:1182-1186 (1971)). Abnormal angiogenesis occurs when the body loses at least some control of angiogenesis, resulting in either excessive or insufficient blood vessel growth. For instance, conditions such as ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing. In contrast, excessive blood vessel proliferation can result in tumor growth, tumor spread, blindness, psoriasis and rheumatoid arthritis.

Thus, there are instances where a greater degree of angiogenesis is desirable-- increasing blood circulation, wound healing, and ulcer healing. For example, recent investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) and Baffour, et al., *J Vasc Surg*, 16:181-91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., *J Surg Res*, 54:575-83 (1993)), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (Takeshita, et al., *Circulation*, 90:228-234 (1994) and Takeshita, et al., *J Clin Invest*, 93:662-70 (1994)).

Conversely, there are instances, where inhibition of angiogenesis is desirable. For example, many diseases are driven by persistent unregulated angiogenesis, also sometimes referred to as "neovascularization." In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries invade the vitreous, bleed, and cause blindness. Ocular neovascularization is the most common cause of blindness. Tumor growth and metastasis are angiogenesis-dependent. A tumor must

continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow.

The current treatment of these diseases is inadequate. Agents which prevent continued angiogenesis, e.g, drugs (TNP-470), monoclonal antibodies, antisense nucleic acids and proteins (angiostatin and endostatin) are currently being tested. See, Battegay, *J. Mol. Med.*, 73, 333-346 (1995); Hanahan et al., *Cell*, 86, 353-364 (1996); Folkman, *N. Engl. J. Med.*, 333, 1757-1763 (1995). Although preliminary results with the antiangiogenic proteins are promising, they are relatively large in size and their difficult to use and produce. Moreover, proteins are subject to enzymatic degradation. Thus, new agents that inhibit angiogenesis are needed. New antiangeogenic proteins or peptides that show improvement in size, case of production, stability and/or potency would be desirable.

SUMMARY OF THE INVENTION

We have now discovered a novel class of antiangiogenic proteins and peptides. Some of the proteins are more potent antiangiogenic agents than presently known proteins such as angiostatin and endostatin. The proteins that can be antiangiogenic agents include those of SEQ ID NOS:1, 2 and 3. We have also discovered pharmaceutical compositions containing an angiogenic inhibitory polypeptide or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.

As used herein an "angiogenic inhibitory polypeptide" refers to a polypeptide having at least one of the following domains: (a) IGF (insulin-like growth factor) binding domain (consens sequence: GCGCCxxC); (b) vWFC (von Willebrand factor type C repeat, Mancuso et al., *J. Biol. Chem.* 264:19514-19527 (1989)); (c) TSP-1 (Thrombospodin type 1 domain,consens sequence: WSxCSccCG); and (d) CTCK-2 (C-terminal cysteine knot profile, Bork P., FEBS 327:125-130(1993)), wherein the polypeptide is an inhibitor of bFGF-stimulated bovine endothelial cell proliferation. That inhibition can be determined by known means such as by using the assay of Folkman et al.(*Natl. Aca. Sci Proc.. USA* 76: 5217-5221, (1979)). Preferably, the protein having at least one of those domains displays those greater than 80%

homology with a protein selected from the group consisting of SEQ ID NO:1 (bovine orthologue for the human connective tissue growth factor, Example 1), SEQ ID NO:2 (human connective tissue growth factor, Bradham et al., *J. Cell. Biol.* 114: 1285-1294 (1991)) and SEQ ID NO:3 (*fisp*-12, Ryseck et al., *Cell Growth Differ.* 2:225-233 (1991)).

In another embodiment, the angiogenic inhibitory polypeptide has at least two of the above listed domains, more preferably three and most preferably all four domains.

Angiogenic inhibitory polypeptides are preferably members of a family of growth regulators referred to as the CCN family. *See, e.g.,* review by Bork P., FEBS 327:125-130(1993). More preferably, the angiogenic inhibitory polypeptides are mammalian connective tissue growth factors.

In a further embodiment, the invention encompasses polypeptides which have at least about 80% identity compared to a mammalian connective tissue growth factor selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 and, more preferably, at least about 85% identity. In more preferred embodiment, this identity is greater than 90%. In a still more preferred embodiment, this identity is greater than 95%.

The invention further relates to treatment of neovascular disorders by administration of a pharmaceutical composition comprising an angiogenic inhibitory polypeptide of the invention or nucleic acid encoding such a polypeptide, and a pharmaceutically acceptable carrier. Such angiogenic inhibitory polypeptides include the mammalian connective tissue growth factor of SEQ ID NO: 1, 2 or 3, and fragments and analogs thereof.

In one embodiment, a pharmaceutical composition of the invention is administered to treat a cancerous condition, or to prevent progression from the pre-neoplastic or pre-malignant state into a neoplastic or a malignant state. In other specific embodiments, a pharmaceutical composition of the invention is administered to treat ocular disorders associated with neovascularization.

Other aspects of the invention are disclosed *infra*.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that the proteins of SEQ ID NOS:1, 2 and 3 display antiangiogenic activity. For example, the protein of SEQ ID NO:1 is more potent than angiostatin as determined by a known assay such as the assay of Folkman et al.(*Proc. Natl. Acc. Sci. USA* 76: 5217-5221, (1979)).

The present invention also relates to therapeutic methods and compositions using "angiogenic inhibitory polypeptides," that have the following properties:

1. have at least one of the following domains: IGF (insulin-like growth factor) binding domain (consens sequence: GCGCCxxC), vWFC (von Willebrand factor type C repeat, Mancuso et al., *J. Biol. Chem.* 264:19514-19527 (1989)), TSP-1 (Thrombospodin type 1 domain,consens sequence: WSxCSccCG) and CTCK-2 (C-terminal cysteine knot profile, Bork P., FEBS 327:125-130(1993));
2. inhibit bFGF-stimulated bovine endothelial cell proliferation using a known assay; and
3. the peptide segment has greater than 80% homology with the corresponding segment of a protein selected from the group consisting of SEQ ID NO:1 (bovine orthologue for the human connective tissue growth factor, Example 1), SEQ ID NO:2 (human connective tissue growth factor, Bradham et al., *J. Cell. Biol.* 114: 1285-1294 (1991)) and SEQ ID NO:3 (*fisp*-12, Ryseck et al., *Cell Growth Differ.* 2:225-233 (1991)).

Homology is determined using the BLAST program provided by GenBank at the National Library of Medicine. GenBank can be accessed via the Internet at www.ncbi.nlm.gov/.

In a preferred embodiment of the invention, the angiogenesis inhibitory polypeptide is a peptide consisting of at least a fragment of SEQ ID NO: 1, 2 or 3, which is effective to inhibit endothelial cell proliferation using the above assay of Folkman et al.

In the above Folkman assay the protein of SEQ ID NO:1 shows a greater potency than angiostatin and endostatin. One can readily determine relative antiangiogenic activity by using the activity of a known antiangiogenic compound and comparing the angiogenic inhibition of the proteins and polypeptides of the present invention.

In another embodiment, the invention encompasses peptides which are homologous to bovine connective tissue growth factor (A1) (SEQ ID NO:1) or fragments thereof. In one embodiment, the amino acid sequence of the peptide has at least 80% identity compared to the fragment of bovine connective tissue growth factor from which it is derived (the "prototype fragment"). In another embodiment, this identity is greater than 85%. In a more preferred embodiment, this identity is greater than 90%. In a most preferred embodiment, the amino acid sequence of the peptide has at least 95% identity with the prototype fragment. Fragments can be at least 10 amino acids, and in preferred embodiments at least 50, 75, 100, 120, and 200 amino acids, respectively.

In another embodiment, the invention encompasses peptides which are homologous to human connective tissue growth factor (SEQ ID NO:2) or fragments thereof. In one embodiment, the amino acid sequence of the peptide has at least 80% identity with the prototype human connective tissue growth factor. In another embodiment, this identity is greater than 85%. In a more preferred embodiment, this identity is greater than 90%. In a most preferred embodiment, the amino acid sequence of the peptide has at least 95% identity with the prototype fragment. Fragments can be at least 10 amino acids, and in preferred 25 embodiments at least 50, 75, 100, 120, and 200 amino acids, respectively.

In another embodiment, the invention encompasses peptides, which are homologous to *fisp-12* (SEQ ID NO:3), the mouse orthologue of human connective tissue growth factor, or fragments thereof. In one embodiment, the amino acid sequence of the peptide has at least 80% identity with the prototype *fisp-12*. In another embodiment, this identity is greater than 85%. In a more preferred embodiment, this identity is greater than 90%. In a most preferred embodiment, the amino acid sequence of the peptide has at least

95% identity with the prototype fragment. Fragments can be at least 10 amino acids, and in preferred embodiments at least 50, 75, 100, 120 and 200 amino acids in length, respectively.

In other specific embodiments, the angiogenic inhibitory polypeptides of the invention are human connective tissue growth factor isoforms from other mammalian species, e.g., rabbit, rat, ovine and porcine.

Angiogenic inhibitory polypeptides of the invention can be combined with a therapeutically effective amount of another molecule which negatively regulates angiogenesis which may be, but is not limited to, platelet factor 4, thrombospondin-1, tissue inhibitors of metalloproteases (TIMP1 and TIMP2) prolactin (16-Kd fragment), angiostatin (38-Kd fragment of plasminogen), endostatin, bFGF soluble receptor, transforming growth factor β, interferon alfa, and placental proliferin-related protein.

An angiogenic inhibitory polypeptide of the invention may also be combined with chemotherapeutic agents.

Connective tissue growth factor protein analogs, can be made by altering the protein sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. These include, but are not limited to, connective tissue growth factor protein analogs, fragments, or analogs containing, as a primary amino acid sequence, all or part of the amino acid sequence of an connective tissue growth factor protein analogs including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members as of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, praline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino

acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

One embodiment of the invention provides for molecules consisting of or comprising a fragment of at least 10 (continuous) amino acids of a connective tissue growth factor protein that is capable of inhibiting endothelial cell proliferation. In other embodiments, this molecule consists of at least 20 or 50 amino acids of the connective tissue growth factor protein. In specific embodiments, such molecules consist of or comprise fragments of a connective tissue growth factor protein of at least 75, 120 or 200 amino acids.

In a preferred embodiment, the protein is a mammalian connective tissue growth factor protein. In alternative embodiments, it is a human, bovine or murine connective tissue growth factor protein. The connective tissue growth factor proteins, fragments and analogs of the invention can be derived from tissue or produced by various methods known in the art. The manipulations, which result in their production, can occur at the gene or protein level. For example, a cloned gene sequence coding for connective tissue growth factor proteins can be modified by any of numerous strategies known in the art. Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d ea., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated in *vitro*. In the production of the gene encoding a derivative or analog, care should be taken to ensure that the modified gene remains within the same translational reading frame as the troponin subunit gene, uninterrupted by translational stop signals, in the gene region where the desired troponin activity is encoded.

The connective tissue growth factors are preferably produced by recombinant methods. See the procedures disclosed in Example 1, which follows. A wide variety of molecular and biochemical methods are available for generating and expressing the polypeptides of the present invention; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols*

in Molecular Biology (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art. For example, the polypeptides of the invention may be obtained by chemical synthesis, expression in bacteria such as *E. coli* and eukaryotes such as yeast, baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of the polypeptide.

The term "isolated" means that the polypeptide is removed from its original environment. For example, a naturally-occurring polynucleotides or polypeptides present in a living animal is not isolated, but the same polynucleotides or DNA or polypeptides, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

Where it is desired to express a polypeptide of the invention any suitable system can be used. The general nature of suitable vectors, expression vectors and constructions therefor will be apparent to those skilled in the art.

Suitable expression vectors may be based on phages or plasmids, both of which are generally host-specific, although these can often be engineered for other hosts. Other suitable vectors include cosmids and retroviruses, and any other vehicles, which may or may not be specific for a given system. Control sequences, such as recognition, promoter, operator, inducer, terminator and other sequences essential and/or useful in the regulation of expression, will be readily apparent to those skilled in the art.

Correct preparation of nucleotide sequences may be confirmed, for example, by the method of Sanger et al. (*Proc. Natl. Acad. Sci.* USA 74:5463-7 (1977)).

A DNA fragment encoding an angiogenic inhibitory polypeptide may readily be inserted into a suitable vector. Ideally, the receiving vector has

suitable restriction sites for ease of insertion, but blunt-end ligation, for example, may also be used, although this may lead to uncertainty over reading frame and direction of insertion. In such an instance, it is a matter of course to test transformants for expression, 1 in 6 of which should have the correct reading frame. Suitable vectors may be selected as a matter of course by those skilled in the art according to the expression system desired.

By transforming a suitable organism or, preferably, eukaryotic cell line, such as HeLa, with the plasmid obtained, selecting the transformant with ampicillin or by other suitable means if required, and adding tryptophan or other suitable promoter-inducer (such as indoleacrylic acid) if necessary, the desired polypeptide or protien may be expressed. The extent of expression may be analyzed by SDS polyacrylamide gel electrophoresis-SDS-PAGE (Lemelli, *Nature* 227:680-685 (1970)).

Suitable methods for growing and transforming cultures etc. are usefully illustrated in, for example, Maniatis (Molecular Cloning, A Laboratory Notebook, Maniatis et al. (eds.), Cold Spring Harbor Labs, N.Y. (1989)).

Cultures useful for production of polypeptides or proteins may suitably be cultures of any living cells, and may vary from prokaryotic expression systems up to eukaryotic expression systems. One preferred prokaryotic system is that of *E. coli*, owing to its ease of manipulation. However, it is also possible to use a higher system, such as a mammalian cell line, for expression of a eukaryotic protein. Currently preferred cell lines for transient expression are the HeLa and Cos cell lines. Other expression systems include the Chinese Hamster Ovary (CHO) cell line and the baculovirus system.

Other expression systems which may be employed include streptomycetes, for example, and yeasts, such as Saccharomyces spp., especially *S. cerevisiae*. Any system may be used as desired, generally depending on what is required by the operator. Suitable systems may also

be used to amplify the genetic material, but it is generally convenient to use *E. coli* for this purpose when only proliferation of the DNA is required.

The polypeptides and proteins may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel electrophoresis; and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

The polypeptides may be generated by any of several chemical techniques. For example, they may be prepared using the solid-phase synthetic technique originally described by R. B. Merrifield, "Solid Phase Peptide Synthesis. I. The Synthesis Of A Tetrapeptide", J. Am. Chem. Soc., 83, pp. 2149-54 (1963), or they may be prepared by synthesis in solution. A summary of peptide synthesis techniques may be found in E. Gross & H. J. Meinhofer, 4 The Peptides: Analysis, Synthesis, Biology; Modern Techniques Of Peptide And Amino Acid Analysis, John Wiley & Sons, (1981) and M. Bodanszky, Principles Of Peptide Synthesis, Springer-Verlag (1984).

The functional activity and/or therapeutically effective dose of an angiogenic inhibitory polypeptide or nucleic acid encoding therefor can be assayed in vitro by various methods. For example, where one is assaying for the ability of the angiogenic inhibitory polypeptides, fragments, and analogs, to inhibit or interfere with the proliferation of capillary endothelial cells (EC) in vitro, various bioassays known in the art can be used, including, but not limited to, radioactive incorporation into nucleic acids, calorimetric assays and cell counting.

Inhibition of endothelial cell proliferation may be measured by calorimetric determination of cellular acid phosphatase activity or electronic cell counting. These methods provide a quick and sensitive screen for determining the number of endothelial cells in culture after treatment with the connective tissue growth factor protein, derivative, or analog of the

invention, and an angiogenesis stimulating factor such as aFGF. The calorimetric determination of cellular acid phosphatase activity is described by Connolly et al., 1986, *J. Anal. Biochem. 152:* 136-140. According to this method, capillary endothelial cells are treated with angiogenesis stimulating factors, such as aFGF, and a range of potential inhibitor concentrations. These samples are incubated to allow for growth, and then harvested, washed, lysed in a buffer containing a phosphatase substrate, and then incubated a second time. A basic solution is added to stop the reaction and color development is determined at 405λ. According to Connolly et al., a linear relationship is obtained between acid phosphatase activity and endothelial cell number up to 10,000 cells/sample. Standard curves for acid phosphatase activity are also generated from known cell numbers in order to confirm that the enzyme levels reflect the actual EC numbers. Percent inhibition is determined by comparing the cell number of samples exposed to stimulus with those exposed to both stimulus and inhibitor.

The incorporation of radioactive thymidine by capillary endothelial cells represents another means by which to assay for the inhibition of endothelial cell proliferation by a potential angiogenesis inhibitor. According to this method, a predetermined number of capillary endothelial cells are grown in the presence of 3H-Thymidine stock, an angiogenesis stimulator such as for example, bFGF, and a range of concentrations of the angiogenesis inhibitor to be tested. Following incubation, the cells are harvested and the extent of thymidine incorporation is determined.

The ability of varying concentrations of angiogenic inhibitory polypeptides to interfere with the process of capillary endothelial cell migration in response to an angiogenic stimulus can be assayed using the modified Boyden chamber technique.

Another means by which to assay the functional activity of angiogenic inhibitory polypeptides involves examining the ability of the compounds to inhibit the directed migration of capillary endothelial cells which ultimately results in capillary tube formation. This ability may be assessed for example, using an assay in which capillary endothelial cells plated on

collagen gels are challenged with the inhibitor, and determining whether capillary-like tube structures are formed by the cultured endothelial cells.

Assays for the ability to inhibit angiogenesis *in vivo* include the chick chorioallantoic membrane assay and mouse, rat or rabbit corneal pocket assays. See, Polverini et al., 1991, *Methods Enzymol.* 198: 440-450. According the corneal pocket assays, a tumor of choice is implanted into the cornea of the test animal in the form of a corneal pocket. The potential angiogenesis inhibitor is applied to the corneal pocket and the corneal pocket is routinely examined for neovascularization.

The therapeutically effective dosage for inhibition of angiogenesis *in vivo*, defined as inhibition of capillary endothelial cell proliferation, migration, and/or blood vessel growth, may be extrapolated from *in vitro* inhibition assays using the compositions of the invention above or in combination with other angiogenesis inhibiting factors. The effective dosage is also dependent on the method and means of delivery. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical-ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant. The protein can also be modified, for example, by polyethyleneglycol treatment.

Diseases, disorders, or conditions, associated with abnormal angiogenesis or neovascularization, and can be treated with a therapuic compound of the invention include, but are not limited to retinal neovascularization, tumor growth, hemagioma, solid tumors, leukemia, metastasis, psoriasis, neovascular glaucoma, diabetic retinopathy, arthritis, endometriosis, and retinopathy of prematurity (ROP).

The term "effective amount" refers to an amount of the angiogenic inhibitory polypeptide the invention sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting inappropriate angiogenesis (neovascularization), limiting tissue damage caused by chronic inflammation, inhibition of tumor cell growth,

and the like. The precise effective amount for a subject will depend upon the subject's size and health, the nature and severity of the condition to be treated, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation based on the information provided herein.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The angiogenic inhibitory polypeptides of the invention are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. Accordingly, angiogenic inhibitory polypeptides of the invention are preferably administered as a pharmaceutical composition comprising an angiogenic inhibitory polypeptide of the invention in combination with a pharmaceutically acceptable carrier. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable carriers (excipients) include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM , vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively one may incorporate or encapsulate the theraputic compound of the invention in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for

implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet Registered TM minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care Registered TM (Allergan), Neodecadron Registered TM (Merck, Sharp & Dohme), Lacrilube Registered TM , and the like, or may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide a theraputic compound of the invention in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

Nucleic acid (DNA) encoding an angiogenic inhibitory polypeptide of the invention can be delivered to a host by any method known to those of skill in the art. For example, catheters, injection, intravenous, parenteral, intraperitoneal and subcutaneous injection, oral or other known routes of administration. The nucleic acid may be delivered "naked" or via a viral vector or liposome.

The amount of angiogenic inhibitory polypeptide of the invention required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The references cited throughout this application are herein incorporated by reference.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Example 1:

Cloning of A1 cDNA from Bovine Aortic Endothelial Cells and Production of Recombinant A1 Protein in Mammalian Cells

A1 cDNA was cloned from bovine aortic endothelial cells by differential screening. A cDNA phage library was prepared from bovine aortic endothelial cells by inserting cDNAs into the EcoRI and Xho I sites of pBluscript vector (Sratagene). Approximately 50,000 plaques were lifted with replica filters for differential hybridization. Probes were prepared by reverse transcription. First, mRNAs were isolated from confluent bovine aortic endothelial (BAE) cells treated with or without 1 nM tumor necrosis factor alpha (TNF-α) for 6 hours. Then one microgram of each mRNA was used to synthesize radioactive cDNA probes of up to $2x10^9$ cpm/μg specific-activity, using M-MuLV reverse transcriptase. Plaques preferentially hybridized with cDNA probe from untreated BAE cells were cloned and further analyzed.

The A1 gene was highly expressed in untreated BAE cells but dramatically down-regulated in TNF-α treated BAE cells. This warranted further studies to look at its antiangiogenic activaties.

The cDNA encoding A1 including the signal peptide was PCR amplified using Taq DNA polymerase. The sequences of the primers were:

5'CTCGAGATGTCAGCCACCGGCCTGGGC3' (SEQ ID NO:4)
5'AAGCTTGGCCATGTCTCCATACATCTT3' (SEQ ID NO:5)

The amplified fragment was then inserted into the Xho I and Hind III sites of pcDNA3.1a, a mammalian expression vector that carries a c-myc epitope for detection and a polyhistidine sequence for purification of the resulted fusion protein (Invitrogen). The sequence of the construct was

confirmed by automatic sequencing. The construct was transfected into 293T cells for transient expression and CHO cells for stable expression of A1 protein. Recombinant A1 protein was isolated from the conditioned media of the above transfected cells by affinity chromatography using HisBind Resin from Novagen.

Example 2:

Recombinant A1 protein inhibits endothelial cell proliferation *in vitro*.

The ability of A1 protein to inhibit growth of endothelial cells was tested by BCE proliferation assay (Folkman et al., *Natl. Aca. Sci Proc.. USA* 76: 5217-5221, 1979). Briefly, cultured bovine capillary endothelial cells dispersed with 0.05% trypsin/0.53 mM EDTA were plated onto gelatinized (Difco) 24-well culture plates (12,500 cell/well) in DMEM containing 10% bovine calf serum (BCS) and incubated for 24 hours. The media was replaced with 0.25 ml DMEM containing 5% bovine calf serum and either buffer only or buffer containing 25 ng/ml to 1 μg/ml of recombinant A1 protein were added. After 20 minutes of incubation, same media containing bFGF were added to obtain a final volume of 0.5 ml and 1 ng/ml bFGF. After 72 hours, the cells were counted with a Coulter Counter.

The recombinant A1 protein potently inhibited endothelial cell proliferation. At concentration of 1 μg/ml, the A1 protein inhibited bFGF driven endothelial cell proliferation by 80%. The IC_{50} (the concentration for half-maximum inhibition) was approximately 50-100 ng/ml. The inhibition was dose dependent and saturable.

Recombinant A1 protein did not inhibit the growth of bovine fibroblasts in culture, indicating that the effect was endothelial cell specific.

Example 3:

A1 protein inhibits tumor angiogenesis and tumor growth *in vivo*

The same construct used for producing recombinant A1 protein *in vitro* was transfected into a human melanoma cell line in culture. Tumor cells that produced recombinant A1 protein were selected and cloned. To

test the effect of A1 protein on tumor angiogenesis and tumor growth *in vivo*, $1x10^6$ human melanoma cells expressing A1 protein or transfected with vector alone were inoculated subcutaneously into the upper-dorsal region of 6-week old male nude mice.

A1 over-expression suppressed the growth of human melanoma by more than 99.9% in nude mice, compared with the tumors transfected with the vector alone (control tumors). From the end of the second week after inoculation, A1 transfected tumors grew to 3-6 mm in diameter and then were held at a static stage, while control tumors continued growing to up to more than ten grams killing the mice. As of the filing date of the present application, A1 transfected tumors were held static for 5 months in one experiment and 6 weeks in another (the experiment was still in progress at the time this application was filed). There was no sign of toxicity. Mice with A1 transfected tumors were healthy and gained weight normally. The long-term inhibition of tumor growth also indicated that there was lack of drug resistance.

Autopsy and histology studies showed that the A1 transfected tumors were disc-shaped pigmented tumors composed of a thin layer of viable tumor cells and a necrotic center. The thickness of viable tumor cell layer was 100-120 μ throughout the entire tumor, which is exactly the effective oxygen diffusion distance. This suggests that the cells beyond this distance could not survive because of the lack of neovascularization in the tumor. In the viable tumor regions, mitotic figures were common but micro vessels were not found. These are typical histological characteristics of tumors suppressed by the inhibition of angiogenesis. Therefore, over expression of A1 protein by tumor cells prevented tumor angiogenesis and consequently, tumor growth *in vivo*.

Over expression of A1 protein in human melanoma cells did not alter cell growth *in vitro*, as compared with the cells transfected with the vector alone. This indicated that the above antitumor effect was not due to an anti-growth effect of A1 protein directly against tumor cells.

Example 4

Functional Domains

Analysis of the A1 peptide sequence revealed 4 functional domains. These are:

Amino acid	34-100:	IGF (insulin-like growth factor) binding domain
		The consensus sequence is GCGCCxxC.
	103-166:	vWFC (von Willebrand factor type C repeat)
		This domain covers cysteines 13-22.
	201-242:	TSP-1 (Thrombospodin type 1 domain)
		The consensus sequence is WSxCSccCG.
	256-330:	CTCK-2 C-terminal cysteine knot profile

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

Claims

What is claimed is:

1. A pharmaceutical composition comprising an amount of a peptide that is effective to inhibit angiogenesis, in which the peptide:

a. has at least one of the following domains: insulin-like growth factor binding domain (consensus sequence: GCGCCxxC), Willebrand factor type C repeat, Thrombospodin type 1 domain (consensus sequence: WSxCSccCG) and C-terminal cysteine knot profile;

b. inhibits bFGF-stimulated bovine endothelial cell proliferation using a known assay; and

c. has greater than 80% homology with the corresponding segment of a protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3;

and a pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the peptide has at least an insulin-like growth factor binding domain and a von Willebrand factor type C repeat domain.

3. The composition of claim 1, wherein the peptide has at least an insulin-like growth factor binding domain, a von Willebrand factor type C repeat domain and a thrombospodin type 1 domain.

4. The composition of claim 1, wherein the peptide has an insulin-like growth factor binding domain, a von Willebrand factor type C repeat domain, a thrombospodin type 1 domain and a terminal cysteine knot profile.

5. The composition of claim 4, wherein the peptide is represented by SEQ ID NO: 1.

6. The composition of claim 4, wherein the peptide is represented by SEQ ID NO: 2.

7. The composition of claim 4, wherein the peptide is represented by SEQ ID NO: 3.

8. The composition of claim 1, wherein the carrier is acceptable for topical application to the skin.

9. A pharmaceutical composition comprising a nucleic acid encoding a peptide that is effective to inhibit angiogenesis, in which the peptide:

a. has at least one of the following domains: insulin-like growth factor binding domain (consensus sequence: GCGCCxxC), Willebrand factor type C repeat, Thrombospodin type 1 domain (consensus sequence: WSxCSccCG) and C-terminal cysteine knot profile;

b. inhibits bFGF-stimulated bovine endothelial cell proliferation using a known assay; and

c. has greater than 80% homology with the corresponding segment of a protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3;

and a pharmaceutically acceptable carrier.

10. A method of inhibiting atopic angiogenesis in a subject, having a disease or disorder causing atopic angiogenesis requiring such inhibition, which comprises administering to the subject of pharmaceutical composition of claim 1 or 9.

11. The method of claim 10, in which the disease or disorder is a solid tumor.

12. The method of claim 11, in which the tumor is a tumor of the central nervous system.

13. The method according to claim 10, in which the disease or disorder is an ophthalmologic disease or disorder.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: CHILDREN'S MEDICAL CENTER CORPORATION

(ii) TITLE OF THE INVENTION: METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Peabody & Brown
(B) STREET: 101 Federal Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 09/119,804
(B) FILING DATE: 21 July 1998

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Resnick, David S
(B) REGISTRATION NUMBER: 34,235
(C) REFERENCE/DOCKET NUMBER: 48217-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-345-6057
(B) TELEFAX: 617-345-1300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 349 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Ser Ala Thr Gly Leu Gly Pro Val Arg Cys Ala Phe Val Leu Leu
 1               5                  10                  15
Leu Ala Leu Cys Ser Arg Pro Ala Ser Ser Gln Asp Cys Cys Ser Ala
            20                  25                  30
Pro Cys Gln Cys Pro Ala Gly Pro Ala Pro Arg Cys Pro Ala Gly Val
        35                  40                  45
Ser Leu Val Leu Asp Gly Cys Gly Cys Cys Val Cys Ala Lys Gln Leu
    50                  55                  60
Ser Glu Leu Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu
65                  70                  75                  80
Phe Cys Asp Phe Gly Ser Pro Thr Asn Arg Lys Ile Gly Val Cys Thr
                85                  90                  95
Ala Lys Asp Gly Ala Pro Tyr Ile Phe Gly Gly Thr Val Tyr Gln Ser
            100                 105                 110
Gly Glu Ser Phe Gln Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp
        115                 120                 125
Gly Ser Val Gly Cys Val Pro Leu Cys Ser Val Asp Val Arg Leu Pro
    130                 135                 140
Ser Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys
145                 150                 155                 160
Cys Glu Glu Trp Val Ser Arg Asp Glu Lys Glu His Thr Val Val Gly
                165                 170                 175
Pro Ala Leu Ala Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro
            180                 185                 190
Thr Met Ile Arg Ala Asn Cys Gln Val Gln Thr Thr Glu Trp Ser Ala
        195                 200                 205
Tyr Ser Lys Thr Cys Gly Met Gly Ile Ser Thr Arg Val Thr Asn Asp
    210                 215                 220
Asn Ala Phe Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg
225                 230                 235                 240
Pro Cys Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Lys Cys
                245                 250                 255
Ile Arg Thr Pro Lys Ile Ser Lys Pro Ile Lys Phe Gln Leu Ser Gly
            260                 265                 270
Cys Thr Ser Met Lys Thr Tyr Arg Ala Lys Phe Phe Gly Val Cys Thr
        275                 280                 285
Asp Gly Arg Cys Cys Thr Pro His Arg Thr Thr Thr Leu Pro Val Glu
    290                 295                 300
Phe Lys Cys Pro Asp Gly Glu Val Met Lys Lys Ser Met Met Phe Ile
305                 310                 315                 320
Lys Thr Cys Ala Cys His Tyr Asn Cys Pro Gly Asp Asn Asp Ile Phe
                325                 330                 335
Glu Ser Leu Tyr Tyr Arg Lys Met Tyr Gly Asp Met Ala
            340                 345
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 349 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Thr Ala Ala Ser Met Gly Pro Val Arg Val Ala Phe Val Val Leu
 1               5                  10                  15
Leu Ala Leu Cys Ser Arg Pro Ala Val Gly Gln Asn Cys Ser Gly Pro
            20                  25                  30
Cys Arg Cys Pro Asp Glu Pro Ala Pro Arg Cys Pro Ala Gly Val Ser
        35                  40                  45
Leu Val Leu Asp Gly Cys Gly Cys Cys Arg Val Cys Ala Lys Gln Leu
    50                  55                  60
Gly Glu Leu Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu
65                  70                  75                  80
Phe Cys Asp Phe Gly Ser Pro Ala Asn Arg Lys Ile Gly Val Cys Thr
                85                  90                  95
Ala Lys Asp Gly Ala Pro Cys Ile Phe Gly Gly Thr Val Tyr Arg Ser
            100                 105                 110
Gly Glu Ser Phe Gln Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp
        115                 120                 125
Gly Ala Val Gly Cys Met Pro Leu Cys Ser Met Asp Val Arg Leu Pro
    130                 135                 140
Ser Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys
145                 150                 155                 160
Cys Glu Glu Trp Val Cys Asp Glu Pro Lys Asp Gln Thr Val Val Gly
                165                 170                 175
Pro Ala Leu Ala Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro
            180                 185                 190
Thr Met Ile Arg Ala Asn Cys Leu Val Gln Thr Thr Glu Trp Ser Ala
        195                 200                 205
Cys Ser Lys Thr Cys Gly Met Gly Ile Ser Thr Arg Val Thr Asn Asp
    210                 215                 220
Asn Ala Ser Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg
225                 230                 235                 240
Pro Cys Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Lys Cys
                245                 250                 255
Ile Arg Thr Pro Lys Ile Ser Lys Pro Ile Lys Phe Glu Leu Ser Gly
            260                 265                 270
Cys Thr Ser Met Lys Thr Tyr Arg Ala Lys Phe Cys Gly Val Cys Thr
        275                 280                 285
Asp Gly Arg Cys Cys Thr Pro His Arg Thr Thr Thr Leu Pro Val Glu
    290                 295                 300
Phe Lys Cys Pro Asp Gly Glu Val Met Lys Lys Asn Met Met Phe Ile
305                 310                 315                 320
```

Lys Thr Cys Ala Cys His Tyr Asn Cys Pro Gly Asp Asn Asp Ile Phe
325 330 335
Glu Ser Leu Tyr Tyr Arg Lys Met Tyr Gly Asp Met Ala
340 345

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Leu Ala Ser Val Ala Gly Pro Ile Ser Leu Ala Leu Val Leu Leu
1 5 10 15
Ala Leu Cys Thr Arg Pro Ala Thr Gly Gln Asp Cys Ser Ala Gln Cys
20 25 30
Gln Cys Ala Ala Glu Ala Ala Pro His Cys Pro Ala Gly Val Ser Leu
35 40 45
Val Leu Asp Gly Cys Gly Cys Cys Arg Val Cys Ala Lys Gln Leu Gly
50 55 60
Glu Leu Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu Phe
65 70 75 80
Cys Asp Phe Gly Ser Pro Ala Asn Arg Lys Ile Gly Val Cys Thr Ala
85 90 95
Lys Asp Gly Ala Pro Cys Val Phe Gly Gly Ser Val Tyr Arg Ser Gly
100 105 110
Glu Ser Phe Gln Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp Gly
115 120 125
Ala Val Gly Cys Val Pro Leu Cys Ser Met Asp Val Arg Leu Pro Ser
130 135 140
Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys Cys
145 150 155 160
Lys Glu Trp Val Cys Asp Glu Pro Lys Asp Arg Thr Ala Val Gly Pro
165 170 175
Ala Leu Ala Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro Thr
180 185 190
Met Met Arg Ala Asn Cys Leu Val Gln Thr Thr Glu Trp Ser Ala Cys
195 200 205
Ser Lys Thr Cys Gly Met Gly Ile Ser Thr Arg Val Thr Asn Asp Asn
210 215 220
Thr Phe Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg Pro
225 230 235 240
Cys Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Lys Cys Ile
245 250 255
Arg Thr Pro Lys Ile Ala Lys Pro Val Lys Phe Glu Leu Ser Gly Cys
260 265 270
Thr Ser Val Lys Thr Tyr Arg Ala Lys Phe Cys Gly Val Cys Thr Asp
275 280 285

```
Gly Arg Cys Cys Thr Pro His Arg Thr Thr Thr Leu Pro Val Glu Phe
    290                 295                 300
Lys Cys Pro Asp Gly Glu Ile Met Lys Lys Asn Met Met Phe Ile Lys
305                 310                 315                 320
Thr Cys Ala Cys His Tyr Asn Cys Pro Gly Asp Asn Asp Ile Phe Glu
                325                 330                 335
Ser Leu Tyr Tyr Arg Lys Met Tyr Gly Asp Met Ala
            340                 345
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTC GAG ATG TCA GCC ACC GGC CTG GGC 27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAC CTT GGC CAT GTC TCC ATA CAT CTT 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13338

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12N 15/00, 15/63; A61K 38/00, 35/00
US CL : 435/69.1, 320.1; 514/2, 44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/69.1, 320.1; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, BIOSIS, WEST, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database EMBL/GENBANK/DDBJ, Accession Number O18739, LILIENSIEK et al., Connective Tissue Growth Factor precursor, 15 July 1998.	1-5, 8-13
X	Database PIR, Accession No. A40551, BRADHAM et al., Connective Tissue Growth Factor - Human. 17 July 1992.	1-4, 6, 8-13
X	Database PIR, Accession No. A53228, RYSECK et al., Fisp-12 Protein Precursor - Mouse, 19 May 1994.	1-4, 7-13
X	Database GENESEQ, Accession No. R79964, BRADHAM et al., Connective Tissue Growth Factor. 12 June 1996.	1-5, 8-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
13 SEPTEMBER 1999	21 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CARRIE STROUP Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13338

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BORK, PEER The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Letters. July 1993, Vol. 327, No. 2, pages 125-130, especially page 126, para 2, page 127, para 3, and page 128, para 5.	1-9
X	HASHIMOTO et al. Expression of the Elm1 Gene, a Novel Gene of the CCN (Connective Tissue Growth Factor, Cyr61/Cefg10, and Neuroblastoma Overexpressed Gene) Family, Suppresses In Vivo Tumor Growth and Metastasis of K-1735 Murine Melanoma Cells. Journal of Experimental Medicine. 02 February 1998, Vol. 187, No. 3, pages 289-296, especially abstract and page 294.	1-9, 11, 12
X	US 5,408,040 A (GROTENDORST et al) 18 April 1995, claim 1.	1-8
X	US 5,780,263 A (HASTINGS et al) 14 July 1998, column 1, lines 62-62, column 6, lines 33-42, column 11, lines 55-62, and column 12, line 7.	8-13